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Morphological traits, DELTA system, and molecular analysis for *Gibellula clavispora* sp. nov. from China

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Abstract — *Gibellula clavispora*, a new fungus collected in Changbai Mountain of Jilin Province, China, is illustrated and described based on morphological traits, the DELTA (DEscription Language for TAxonomy) system, and molecular analysis. *Gibellula clavispora* is characterized by cylindrical synnemata, aspergillate conidiophores with an apical vesicle, obovoid metulae (8.6–10.8 × 2.2 µm), clavate phialides (5.4–6.5 × 1.1–2.2 µm), distinctly clavate conidia, and the absence of *Granulomanus*-synanamorphic conidiogenous cells.

Key words — araneogenous fungus, numerical classification, phenotypic traits, phenetic tree, Splitstree

Introduction

Cavara (1894:) introduced the genus *Gibellula* with *G. pulchra* Cavara as the type species. *Gibellula* species are highly specialized and apparently obligate parasites of spiders with a worldwide distribution (Samson & Evans 1992). Since the genus was established, several more species have been described on spiders (Petch 1932; Mains 1950; Samson & Evans 1973, 1977; Humber & Rombach 1987). When Samson & Evans (1992) described four new *Gibellula* species from infected spiders collected in South America, they thoroughly discussed the taxonomy, host specificity, habitats, and teleomorph connections and presented a key to the nine accepted *Gibellula* species. Tzean et al. (1997, 1998), who conducted an extensive study of the insect and spider pathogens from Taiwan since 1989, described three new *Gibellula* taxa, including *G. clavulifera* var. *major*, *G. unica*, and *G. dimorpha*. Although more than 30 names have been published, most are treated as synonyms (http://www.indexfungorum.org/Names/Names.asp).

After a survey of the synnematous entomogenous fungi in China, we reported 15 species of *Gibellula* (Chen et al. 2014), using morphological characters as the main identification criteria in the absence of any significant body of gene sequences in GenBank or other genomic databases. In order to make the taxonomy of *Gibellula* more effective and rational, additional taxonomic approaches were required for identifying a new species in *Gibellula*.

DELTA (DEscription Language for TAxonomy), a computer program developed for botanists (Dallwitz 1980) to generate taxonomic output and interactive identification, is not yet widely used by mycologists. The morphology-based DELTA expert system offers many advantages over the more traditional taxonomic approaches for animals, plants, and microbes because it uses digital standardization of morphological characters to determine which are suitable for taxonomic identification (Li et al. 1993; Li 1996; Chang et al. 2000; Carney 2003; Chen & Chen 2008; Han et al. 2009, 2010) and international communication (Chen et al. 2000a,b). We have been successful in classifying *Gibellula* species using the DELTA system (Chen et al. 2014).

In this paper, we describe and illustrate a new *Gibellula* species, *Gibellula clavispora*, which we identified by combining a classical morphological approach with the DELTA system and ITS sequence analysis.

Materials & methods

Collection and morphological examination

A *Gibellula* specimen (GZUIF07815) was obtained from a naturally infected spider collected on Changbai Mountain, Jilin Province, China, in July 2007. The specimen was oven dried at 40°C to prevent growth of fungal contaminants. Snippets of outer layer tissue from the delaminated synnemata in the dried specimen were mounted in lactophenol cotton blue solution and examined morphologically under a light microscope following procedures set forth by Liang et al. (2005). The fungus is deposited in the Institute of Fungal Resource, Guizhou University, Guiyang, China (GZUIF).

DNA extraction, amplification, and sequencing

DNA was extracted from the specimen according to Tigano-Milani et al. (1995) and stored at –20°C. Taq enzyme and dNTP were obtained from Shanghai Sangon. The internal transcribed spacer region (ITS1+5.8S rDNA+ITS2) was amplified by polymerase chain reaction (PCR) using the primers ITS5 (5′-GGTGAGAGATTTCTGTGC-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′)(Han et al. 2013). After initial denaturation at 94°C for 5 min, the DNA was amplified for 35 cycles with denaturation at 94°C for 40 s, annealing at 49°C for 40 s, and extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min. The PCR products were purified using the UNIQ-10 column PCR product purification kit (no. SK1141; Sangon Biotech Co., Shanghai, China) according

Table 1. Specimens and ITS sequences used in the phylogenetic analysis
(New sequences are in bold font.)

Taxon	Specimen/Strain	Country	GenBank
Isaria farinosa (Holmsk.) Fr.	CBS 262.58	Thailand	AY624179
Gibellula clavispora	GZUIF07815	China	**KJ857270**
G. clavulifera var. *clavulifera*	HN0801	China	**KP685596**
G. leiopus	GZUIFR–XS0805	China	**KP685597**
G. pulchra	XCH0827	China	**KP685595**
Gibellula sp.	BCC14505	Thailand	GQ250021
Gibellula sp.	NHJ13158	USA	JN049864
Gibellula sp.	BCC02757	Thailand	GQ250020
Gibellula sp.	BCMU GP01	Japan	AB237661
Gibellula sp.	MY2566	Thailand	HM161738
Torrubiella arachnophila	NHJ14150	Thailand	HM161739
T. arachnophila	EPF083	Thailand	JX192719
T. flava	NBRC 30612	Japan	AB100609
T. ratticaudata	ARSEF 1915	USA	JN049837

to the manufacturer's protocol and sequenced with ITS5 and ITS4 at Sangon Biotech Co. The full ITS sequence (GZUIF07815) was deposited as GenBank KJ857270.

Sequence alignment and phylogenetic analyses

Sequence data from the holotype specimen of the new species were compared with ITS sequences obtained from GenBank. Representative sequences with similarities of >90% are included in our analysis. Full ITS sequences from selected known allies were also downloaded from GenBank (Benson et al. 2000). These sequences (Table 1) were aligned by the CLUSTAL W program (Thompson et al. 1997) using the default settings followed by manual refinements.

The sequences were analyzed by neighbor-joining (NJ) methods (Saitou & Nei 1987), maximum parsimony (MP), and maximum likelihood (ML) methods using MEGA ver. 5 (Tamura et al. 2011). NJ analysis used the default parameters, with distance values of samples calculated by the Kimura two-parameter model (Kimura 1980). ML analysis followed the GTR+G+I model (six general time reversible substitution rates, assuming gamma distributed with invariant sites). The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm (Nei & Kumar 2000) with search level 1, in which the initial trees were obtained with the random addition of sequences (ten replicates). Bootstrap values of the three methods were obtained from 1000 replications per analysis. Other parameters followed the default conditions. *Isaria farinosa* (Holmsk.) Fr. was designated as outgroup.

A network was created by Splitstree version 4.10 (Huson & Bryant 2006) using the sequences from the phylogenetic tree except for the outgroup. The running conditions were *P*-distance, Unrooted Neighbor Net.

DELTA system

Fifteen *Gibellula* species were used in the study (TABLE 2). A data matrix (TABLE 3) was generated using published character data from species descriptions (Chen et al. 2014). These characters were then used to generate a phenetic tree (FIG. 3; Chen et al. 2014) in DELTA using the PCLASS program in accordance with the program CONFOR using the Editor "todis" directives.

TABLE 2. *Gibellula* spp. used for constructing the DELTA database

TAXON
G. alata Petch
G. brunnea Samson & H.C. Evans
G. clavata Samson & H.C. Evans
G. clavispora
G. clavulifera var. *alba* Humber & Rombach
G. clavulifera (Petch) Samson & H.C. Evans var. *clavulifera*
G. clavulifera var. *major* Tzean et al.
G. curvispora Z.Q. Liang et al.
G. dabieshanensis B. Huang et al.
G. dimorpha Tzean et al.
G. leiopus (Vuill. ex Maubl.) Mains
G. mainsii Samson & H.C. Evans
G. mirabilis Samson & H.C. Evans
G. pulchra Cavara
G. unica L.S. Hsieh et al.

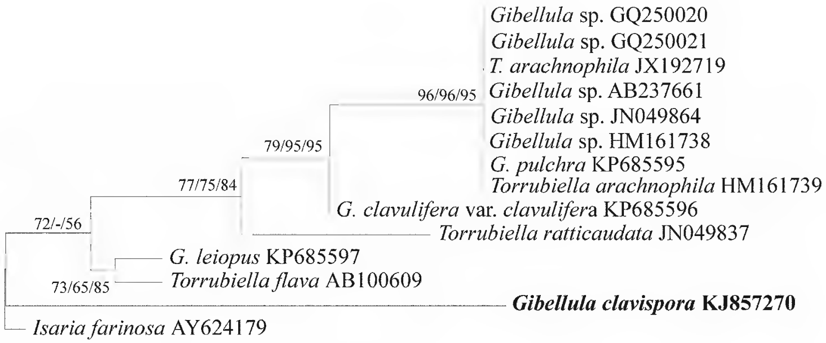
TABLE 3. Phenetic characters and their states for species of *Gibellula*.

1. Host	a. spider; b. insect
2. Synnemata	a. present; b. absent
3. Synnema state	a. solitary; b. in pairs; c. scattered; d. clustered
4. Synnema shape	a. clavate; b. cylindrical
5. Synnema tip	a. bulbous; b. acuminate
6. Conidiophores	a. penicillate; b. aspergillate
7. Conidiophore surface	a. smooth; b. rough
8. Conidiophores bases	a. pigmented; b. hyaline
9. Conidiophore length	(µm)
10. Vesicle	a. present; b. absent or hardly developed
11. Metulae shape	a. ovoid; b. ellipsoidal; c. obovoid; d. clavate; e. cylindrical
12. Phialide shape	a. clavate; b. cylindrical; c. ellipsoidal
13. Phialide neck	a. present; b. absent
14. Phialide length	(µm)
15. Phialide width	(µm)
16. Conidium shape	a. clavate; b. ovoid; c. allantoidal; d. fusiform; e. ellipsoid
17. Conidium surface	a. smooth; b. apiculate
18. Conidium length	(µm)
19. Conidium width	(µm)
20. *Granulomanus* synanamorph	a. present; b. absent
21. *Granulomanus* conidiophores	a. rough; b. smooth

Results

Phylogenetic analysis

For MP analysis of the 14-taxon ITS dataset (150 positions), the consistency index was 0.892857, the retention index was 0.911756, and the composite index was 0.814076 (0.785714) for all sites (parsimony-informative sites). ML analysis of the same dataset produced a tree with a log likelihood of –349.60. The ITS-based phylogram (Fig. 1) clearly supports the new species, *Gibellula clavispora*, in an independent clade.



Gibellula sp. GQ250020
Gibellula sp. GQ250021
T. arachnophila JX192719
96/96/95
Gibellula sp. AB237661
Gibellula sp. JN049864
79/95/95
Gibellula sp. HM161738
G. pulchra KP685595
77/75/84
Torrubiella arachnophila HM161739
G. clavulifera var. *clavulifera* KP685596
72/-/56
Torrubiella ratticaudata JN049837
G. leiopus KP685597
73/65/85
Torrubiella flava AB100609
***Gibellula clavispora* KJ857270**
Isaria farinosa AY624179

Fig. 1. Phylogenetic tree generated based on the ITS sequences. Statistical support values (≥50%) are shown at nodes, for neighbor-joining/maximum parsimony/maximum likelihood.

Taxonomy

Gibellula clavispora Z.Q. Liang, Wan H. Chen & Y.F. Han, **sp. nov.** Fig. 2

MycoBank MB 810567

> Differs from other *Gibellula* species by its cylindrical synnemata, aspergillate conidiophores, obovoid metulae, clavate phialides, and distinctly clavate conidia.
>
> Type: China, Jilin Province, Changbai Mountain, spider, 15 August 2007, A.Y. Liu (**Holotype**, GZUIF07815).
>
> Etymology: referring to the shape of conidia.

Host: a species of spider completely covered by white mycelium. Synnemata solitary, arising from the host, cylindric, slender, scattered, attenuated, and composed of parallel, multiseptate longitudinal hyphae. Hyphae branched, hyaline, smooth. Conidiophores hyaline, septate, arising from aerial mycelium or synnemata, scattered, 96–113 µm long, septa conspicuous, smooth or occasionally roughened along the length, narrowing abruptly to a slender apex, and terminating in a swollen vesicle. Vesicle obovate, hyaline, smooth-walled. Phialides clavate, smooth-walled, with a short neck, hyaline,

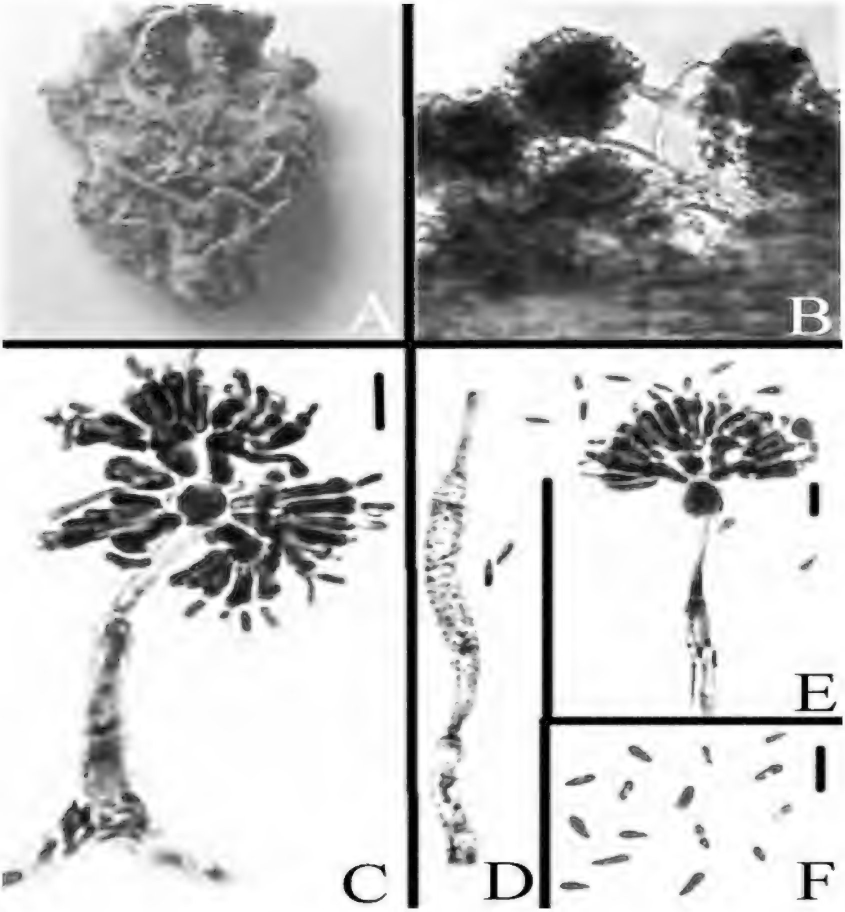


Fig. 2. *Gibellula clavispora* (holotype, GZUIF07815) A: infected spider; B: conidiophores arising from the synnema; C–E: conidiophores with smooth or occasionally rough surface; F: conidia. Scale bars: C, E, F = 10µm.

8.6–10.8 × 2.2 µm. Metulae obovate, hyaline, smooth-walled, 7.6–8.6 × 3.2 µm, borne on vesicle. Conidia clavate, hyaline, smooth-walled, single, 5.4–6.5 × 1.1–2.2 µm, occasionally ellipsoidal, 2.2–4.3 × 1.1 µm. Neither teleomorph nor *Granulomanus* synanamorph observed.

Comments: Three other *Gibellula* species produce clavate conidia—*G. alata*, *G. brunnea*, and *G. mainsii* (Table 4). *Gibellula alata* is distinguished by its short slender synnemata terminating in a bulbous outgrowth from which a number of conidiophores and diagnostic wing-like structure arise. *Gibellula brunnea*

TABLE 4. Morphological comparison between *Gibellula clavispora* and allied species.

SPECIES	SYNNEMA APEX	*GRANULOMANUS* SYNANAMORPH	PHIALIDE SHAPE & SIZE (µm)	CONIDIAL SHAPE & SIZE (µm)
G. alata	bulbous	absent	clavate, 9–13 × 3–4	oblong-oval or clavate, 4–9 × 2–4
G. brunnea	acuminate	present	cylindrical, 10–13 × 3–4	fusiform or sl. clavate, 8–10 × 2–2.5
G. clavispora	acuminate	absent	clavate, 8.6–10.8 × 2.2	clavate, 5.4–6.5 × 1.1–2.2
G. mainsii	acuminate	absent	cylindrical, 10–13 × 3–4	fusiform or sl. clavate, 8–10 × 2–2.5

differs from *G. clavispora* in its *Granulomanus* synanamorph state, while *G. mainsii* differs in its cylindrical phialides.

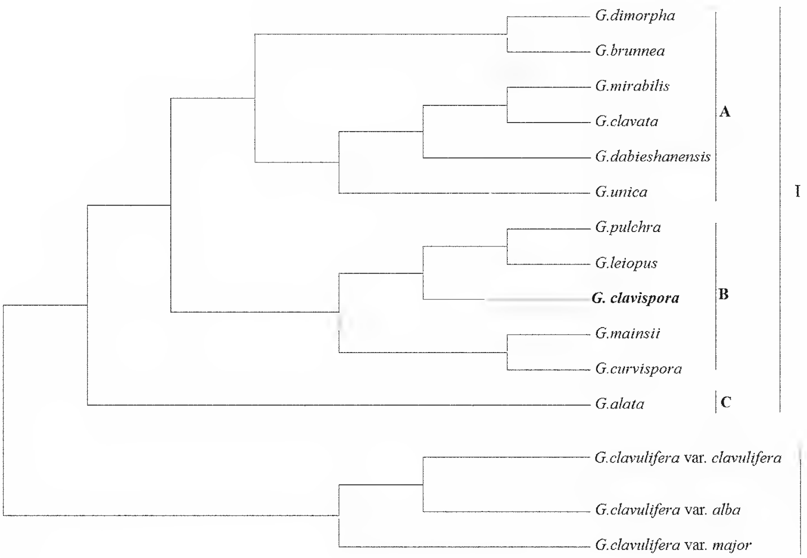


FIG. 3. Phenetic tree generated for *Gibellula* spp. by DELTA.

DELTA system analysis

DELTA system analysis is a numerical character classification. It was not granted special priority, so that all characters selected were weighted equally in this study. The phenetic tree (FIG. 3) based on the morphological characteristics presented in TABLE 3, separates *Gibellula* into several distinct groups. The new species *G. clavispora* was grouped together with *G. pulchra*, *G. leiopus*, *G. mainsii*, and *G. curvispora* on a branch nested within the other species of this genus.

Discussion

The solitary septate conidiophores of this new species are scattered on the host body or synnemata, usually with small but distinct terminal vesicle bearing one or two orders of the tightly clustered metulae characteristic of *Gibellula* species (Humber & Rombach 1987). *Gibellula* is further distinguished from allied genera by its spider-specific species characterized by septate conidiophores that narrow abruptly to a slender apical neck (Samson et al. 1992). It is obvious that *G. clavispora* belongs in *Gibellula* and represents a new species morphologically distinguished by cylindrical synnemata, aspergillate conidiophores, obovoid metulae, clavate phialides, distinctly clavate conidia, and the absence of conidiogenous cells of *Granulomanus* synanamorph.

The DELTA system analysis produced a clustering diagram dividing *Gibellula* into two distinct groups (I–II) and three subgroups (A–C). The two groups are separated by aspergillate (Group I) and penicillate (Group II) conidiophores, which is consistent with the findings of Tzean et al. (1997).

Group II taxa—*G. clavulifera* var. *clavulifera*, *G. clavulifera* var. *alba*, *G. clavulifera* var. *major*—are characterized by solitary and attenuated synnemata, penicillate conidiophores, and the absence of vesicles.

Group I is divided into three subgroups. Subgroup A, comprising *G. dimorpha*, *G. mirabilis*, *G. clavata*, *G. dabieshanensis*, and *G. unica*, is characterized by solitary or paired, attenuated synnemata, aspergillate conidiophores, cylindrical phialides, and fusiform conidia. Subgroup B (*G. pulchra*, *G. leiopus*, *G. clavispora*, *G. mainsii*, *G. curvispora*, and *G. brunnea*) is characterized by cylindrical and scattered synnemata and the absence of a *Granulomanus* synanamorph. Subgroup C, containing only *G. alata*, is distinguished by the flattened-globose head and wing-like extension at the terminal apex of its synnemata.

DELTA system analysis revealed that some characters, such as conidiophore shape and the presence/absence of vesicle and *Granulomanus* synanamorph, could help us conveniently identify species. The phenetic tree places the new species *G. clavispora* on an independent branch, as separated by its clavate conidia, aspergillate conidiophores, and absence of *Granulomanus* synanamorph.

The ITS-derived phylogram (Fig.1) also placed *G. clavispora* in an independent clade. The phylogenetic and phenetic trees were not congruent, possibly due to inherent differences between morphological and molecular characters.

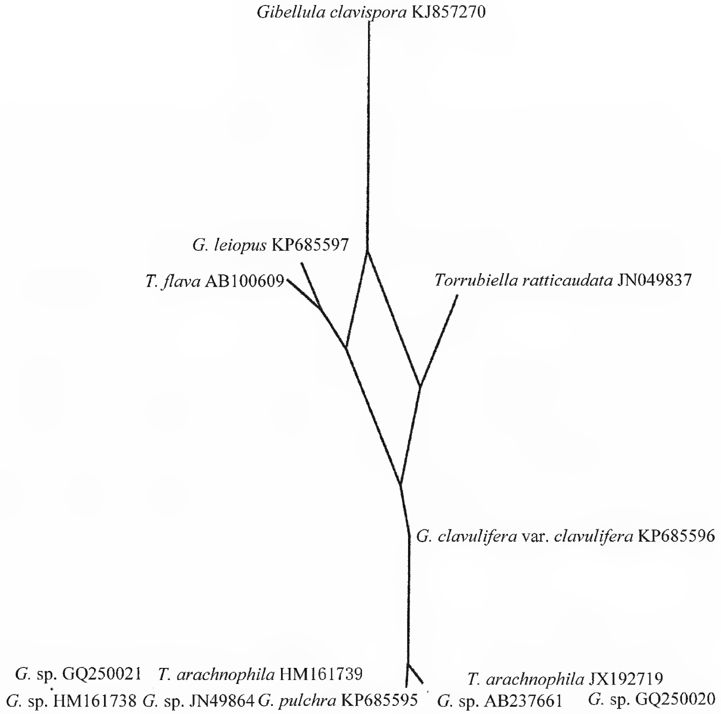

Gibellula clavispora KJ857270
G. leiopus KP685597
T. flava AB100609
Torrubiella ratticaudata JN049837
G. clavulifera var. *clavulifera* KP685596
G. sp. GQ250021
T. arachnophila HM161739
T. arachnophila JX192719
G. sp. HM161738
G. sp. JN49864
G. pulchra KP685595
G. sp. AB237661
G. sp. GQ250020

Fig. 4. Reconstruction of the Neighbor-Net network from taxa in Fig. 1.

We investigated the incongruence in the horizontal processes using Splitstree4 (Huson & Bryant 2006), which produced a recomputed neighbor-net (Fig.4) with a 99.999 fit and 99.999 LS fit. The result revealed an ambiguous signal present between *G. clavispora* and other species, with *G. clavispora* on a separate branch in the neighbor-net. Combining morphology and phylogenetic tree, Neighbor-Net analyses support *Gibellula clavispora* as a new taxon in *Gibellula*.

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